Evaluation of Neuroprotective Effects of Sinomenine in Rotenone-Induced Parkinson's Disease in *Drosophila Melanogaster*

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ABSTRACT

Background: Parkinson's disease (PD) ranks as the second most common neurodegenerative disorder, primarily impacting the elderly population. It is caused by degeneration of dopaminergic neurons in the striatum. Sinomenine (SN) has been shown todecrease the production of pro-inflammatory mediators, such as intracellular ROS, TNF-alpha, NO, and PGE2 thereby reducing neuroinflammation. Thus, the study was designed to assess the neuroprotective effect of SN in the rotenone-induced PD of *Drosophilamelanogaster*.

Materials and Method: *Drosophila* flies were cultured in cornmeal agar medium. Seven-day-old flies were divided into five groups (n=30): normal control, disease control, Levodopa (1 mM), and SN low (0.125%) and high dose (0.25%). Rotenone (125 µM) was used to induce the disease. All drugs were administered through the cornmeal agar medium for seven days, and on the eighth day, a climbing assay was performed. Effect on biochemical variables like Malondialdehyde (MDA) and Dopamine levels in the *Drosophila* brain were also assessed.

Result: There was a significant improvement in the flying and climbing ability of *Drosophila* flies in the SN (high and low doses) and L-dopa groups compared to the disease control group. Also, there was a significant increase in levels of Dopamine and a decrease in levels of MDA in the *Drosophila* brain in the SN-treated groups and the L-dopa group. All these effects were most pronounced in the group receiving a high dose of SN.

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Conclusion: Sinomenine was found to be neuroprotective as it improved the locomotor activity of *Drosophila* flies. It acts by preventing the degeneration of dopaminergic neurons, probably by reducing oxidative stress.

Keywords: Dopamine, Malondialdehyde, Levodopa, Rotenone, Climbing assay
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INTRODUCTION

Parkinson's disease (PD) stands as the second most prevalent neurodegenerative condition, following Alzheimer's disease, and it primarily impacts the elderly population. Bradykinesia, resting tremors, rigidity, gait disturbances, masked facies, decreased blink frequency and swallowing difficulties are some of the symptoms experienced by the patients.[1] In terms of its pathophysiology, PD is marked by the significant loss, ranging from approximately 50% to 70%, of dopaminergic neurons within the substantia nigra pars compacta. It leads to the deficiency of dopamine in the corpus striatum and the accumulation of intraneuronal protein inclusion bodies known as Lewy bodies.[2,3] Earlier research has indicated that the development of PD may be influenced by factors such as oxidative stress, the formation of free radicals, genetic predisposition, and programmed cell death.[4] A frequently used pesticide, Rotenone has a strong affinity for the electron transport chain within mitochondria. This interaction leads to mitochondrial disruptions, dose-dependent depletion of ATP, oxidative harm, and premature mortality of dopaminergic neurons, mirroring the characteristics of PD. Exposing flies to rotenone via agar for seven days can induce impaired locomotion and loss of dopaminergic neurons which resembles PD.[5]

Presently, the pharmacological treatments accessible for PD can only temporarily stall its advancement, lacking the ability to impede or reverse the disease's progression. Levodopa is the most effective drug in the treatment of PD but often provides incomplete relief, especially after its chronic use. It is unable to halt the disease's progress and patients experience wearing-off phenomena, dyskinesias, freezing episodes, and unpredictable "on-off" fluctuations, which pose significant challenges.[6]

Sinomenine (SN) is an alkaloid derived from the Chinese medicinal plant *Sinomenium acutum*. It has been used for millennia to treat inflammatory diseases.[7] The suppression of microglial generation of superoxide proved to be highly successful in protecting DA neurons in the culture.[8] According to reports, SN hinders the generation of pro-inflammatory substances such as TNF-α, IL-1, PGE2, leukotriene C4, and NO by macrophages.[9] Hence, the study was planned to evaluate the neuroprotective potential of Sinomenine in the rotenone-induced Parkinson's disease model in *Drosophilamelanogaster*.

MATERIALS AND METHOD

The work was carried out in two models: rotenone-induced PD in the *Drosophila* model and 6-hydroxydopamine (6-OHDA) models in the rats. Prior approval from the institutional ethics committee was obtained before commencing the study. The findings from the rat model study will be published in a separate publication.

Drosophila melanogaster: Its wild strain (Canton Special) was obtained from the Tata Institute of Fundamental Research (TIFR), Colaba, Mumbai.

Study drugs/ chemicals:

- Sinomenine (SN): The doses used were 0.25% & 0.125%, obtained from a dose-finding study.
- Viability assay (Dose finding study):- Since we could not find any study of SN in rotenone induced PD model of *Drosophila melanogaster*, we conducted a dose-finding study to select appropriate doses for the experiment. Study drugs used in the experiment are administered by mixing them in fly food (culture medium). 7-day-old adult *Drosophila* flies were used for standardization purposes. They

were divided into 5 groups of 30 flies each and SN in concentrations of 2%, 1%, 0.5%, 0.25% & 0.125 % were added to their food. The exposure of SN was given for 1-2 days and it was observed that there was significant mortality (>50%) in the groups receiving SN doses of 0.5% & more, hence lower concentrations i.e., 0.25% & 0.125% were chosen for the study.

- Rotenone: It was used as an inducing agent in a dose of 125 µM. It was dissolved in Dimethyl sulfoxide (DMSO).[10]
- Levodopa (L-Dopa): It was used as a positive control in a dose of 1mM.[11]

All the chemicals were acquired from Sigma Aldrich.

Other resources:

- Cornmeal agar medium: It was prepared as per the instructions of Lewis laboratories. The recipe yields around 18 liters of cornmeal agar by combining 17 liters of distilled water with 93 grams of agar, 1,716 grams of cornmeal, 310 grams of Baker's Yeast, 517 grams of sugar, 1,033 grams of Dextrose, and 200 milliliters of acid mix.[12]
- Glass bottles: Their openings were covered with cotton plugs.
- Glass cylinders: 30 cm tall with a diameter of 2 cm, closed at one end.

Study Groups: There were 5 study groups, and they are divided as follows: **(Table1)**

Table 1: Study groups

Sr. No	Groups	Inducing agent	Drugs
1.	Normal Control (NC)	-	-
2.	Disease Control (DC)	Rotenone 125µM	-
3.	L-Dopa (LD)		L-Dopa (1 mM)
4.	Sinomenine Low dose (SN-L)		Sinomenine (0.125%)
5.	Sinomenine High dose (SN-H)		Sinomenine (0.25%)

(n=30)Theinducing agent and drugs were given for seven days through cornmeal agar medium.

Procedure

Drosophilamelanogaster culture was initiated in a glass container filled with a cornmeal-based medium. Once they reached an age of approximately 5-7 days, they were separated into a new glass bottle containing cornmeal agar medium. After that, 30 flies each were transferred to all study groups mentioned above. Then, the inducing agent and study drugs were given through cornmeal agar for seven days. After seven days, these flies of different groups were transferred into separate graduated cylinders 30 cm tall and 2 cm diameter with a middle mark at 15 cm and a lid to carry out a climbing assay to assess motor coordination. Behavioral assessment was done using a climbing assay.

Climbing Assay: It was done to assess the locomotor ability. The flies were sorted under brief ice anesthesia. After 20 minutes, cylinders were tapped so that the flies settled in the bottom and then allowed to reach the top of the cylinder. The flies that reached the top, i.e., beyond the 15 cm mark and those that remained at the bottom were counted separately after 45 seconds. The procedure was repeated three times.[13,14]

Biochemical Assay

MDA & Dopamine estimation: After the climbing assay, the heads (brains) of *Drosophila* flies were separated and crushed in mortar and pestle, and the homogenate was prepared. For this, the flies were immobilized by chilling on ice and then decanted into a chilled mortar. The

heads of flies were homogenized in ice-cold Tris/ HCl buffer (pH 7.4, 0.1 M), 1:10 (flies/volume µL). The homogenate was filtered through a sieve with nylon mesh (pore size/10 mm), centrifuged at 3000 x g for 3 minutes, and the supernatant was used for biochemical assays (20 flies/ 200 µL). The malondialdehyde (MDA) and dopamine levels were estimated using ELISA from that homogenate.[15,16]

Statistical analysis: Data were analyzed by one-way analysis of variance (ANOVA) followed by a post-hoc Tukey test using GraphPad Prism. The significance level was decided as $p<0.05$, and the outcomes were presented as mean ± SD.

RESULTS

The objective of this study was to evaluate the neuroprotective potential of Sinomenine (SN). The findings suggest that SN, administered in 0.125% and 0.25% doses showed neuroprotective effects against rotenone-induced Parkinson's disease in *Drosophilamelanogaster*.

SN showed a significant increase in the climbing ability in both high-dose and low-dose groups compared to the disease control group. Moreover, SN, at a high dose of 0.25%, showed a significant increase in climbing ability compared to the standard control L-dopa group. (**Table 2** &**Figure 1**)

Table 2: Effect on Climbing Activity

Sr No	Groups	Number of flies crossing midline (Mean ± SD)
1	Normal control (NC)	30 ± 0
2	Disease control (DC)	9.33 ± 0.47 *
3	Levodopa (LD)	22.67 ± 0.94 #
4	Sinomenine Low dose (SN-L)	16.33 ± 1.25 #
5	Sinomenine High dose (SN-H)	19.67 ± 1.25#

n=30, values expressed as mean ± SD. * = p< 0.001 vs Normal control, # = p< 0.001 vs Disease control. (One-way ANOVA followed by post hoc Tukey's test)

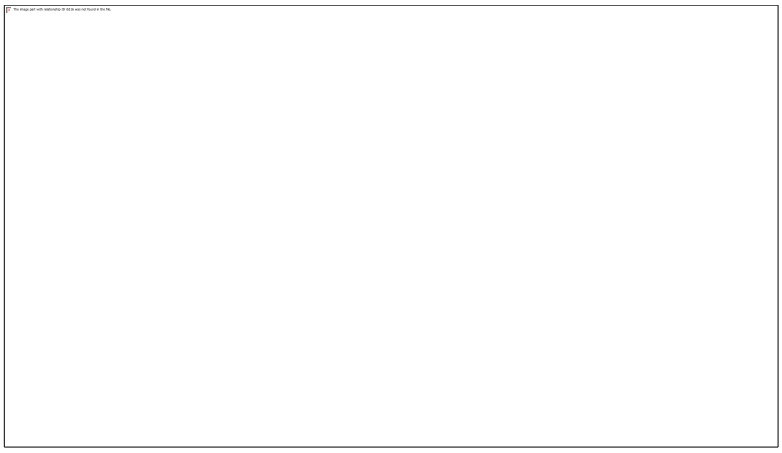

Figure 1: Effect on climbing activity

n=30, values expressed as mean ± SD. * p< 0.001 vs Normal control, # p< 0.001 vs Disease control. (One-way ANOVA followed by post hoc Tukey's test)

(NC = Normal control, DC = Disease control, LD = Levodopa, SN-L = Sinomenine Low dose, SN-H = Sinomenine High dose)

In the biochemical assay, MDA and dopamine levels were assessed through the ELISA method.

The level of MDA decreased significantly in the levodopa group followed by the SN high-dose group as compared to the disease control group. The fall in MDA in the SN low-dose group was not statistically significant **(Table 3 & Figure 2).**

Table 3: Effect on Brain Malondialdehyde (MDA) Concentration (nmoles/ml)

Sr No	Groups	Mean ± SD
1	Normal control (NC)	1.9 ± 0.09
2	Disease control (DC)	3.58 ± 0.12 *
3	Levodopa (LD)	3.22 ± 0.06 #
4	Sinomenine low dose (SN-L)	3.42 ± 0.15
5	Sinomenine high dose (SN-H)	3.29 ± 0.11 @

n=30, values expressed as mean ± SD. * = p< 0.001 vs Normal control, # = p< 0.001 vs Disease control, @ = p< 0.01 vs Disease control. (One-way ANOVA followed by post hoc Tukey's test)
(NC = Normal control, DC = Disease control, LD = Levodopa, SN-L = Sinomenine Low dose, SN-H = Sinomenine High dose)

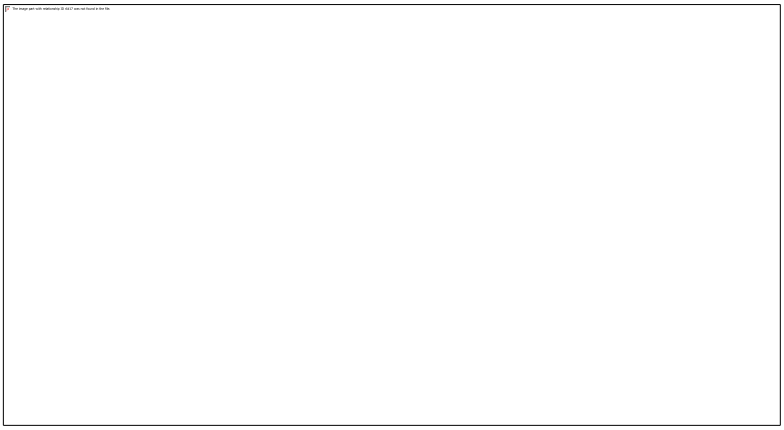

Figure 2: Effect on Brain Malondialdehyde (MDA) Concentration (nmoles/ml)

n=30, values expressed as mean ± SD. * = p< 0.001 vs Normal control; # = p< 0.001 vs Disease control, @ = p< 0.01 vs Disease control. (One-way ANOVA followed by post hoc Tukey's test)
(NC = Normal control, DC = Disease control, LD = Levodopa, SN-L = Sinomenine Low dose, SN-H = Sinomenine High dose)

In Dopamine level estimation, it was found that the level of dopamine improved maximally in the Levodopa group followed by SN high-dose&SN low-dose groupswhen compared with the disease control. **(Table 4 & Figure 3)**.

Table 4: Effect on Brain Dopamine Levels (ng/ml)

Sr No	Groups	Mean ± SD
1	Normal control (NC)	1.125 ± 0.008
2	Disease control (DC)	0.67 ± 0.01 *
3	Levodopa (LD)	0.94 ± 0.008 #
4	Sinomenine low dose (SN-L)	0.85 ± 0.008 #
5	Sinomenine high dose (SN-H)	0.915 ± 0.007 #

n=30, values expressed as mean ± SD. * p< 0.001 vs Normal control; # p< 0.001 vs Disease control. (One-way ANOVA followed by post hoc Tukey's test)

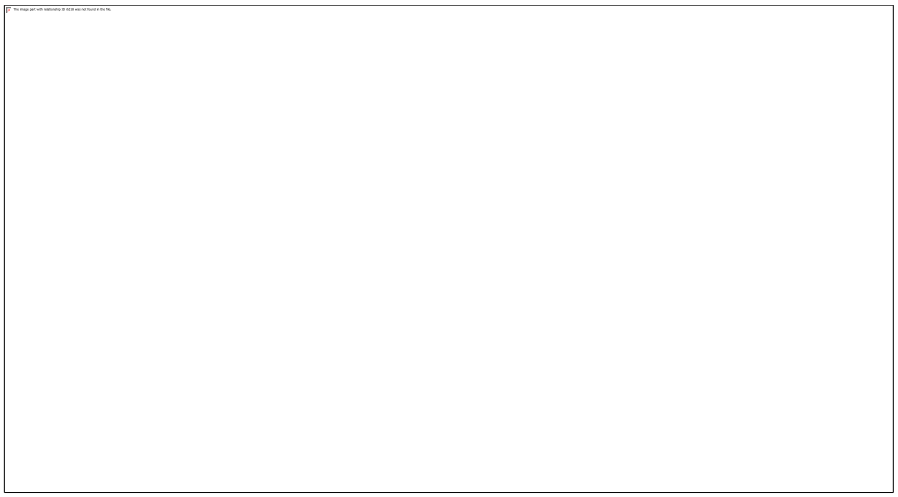

Figure 3: Effect on Brain Dopamine Levels (ng/ml)

n=30, values expressed as mean ± SD. * p< 0.001 vs Normal control; # p< 0.001 vs Disease control. (One-way ANOVA followed by post hoc Tukey's test)

(NC = Normal control, DC = Disease control, LD = Levodopa, SN-L = Sinomenine Low dose, SN-H = Sinomenine High dose)

DISCUSSION

In the present study, we explored the neuroprotective effects of Sinomenine (SN) in a *Drosophila* insect system, which is widely utilized for simulating neurodegenerative disorders. *Drosophila* when exposed to small quantities of rotenone produces features of Parkinson's disease (PD) like locomotor impairments and decline in dopaminergic neurons.[10] The primary advantage of this model is the ability to quickly screen potential therapeutic agents or phytochemicals for various neurodegenerative disorders.[13,14] But, the exact mechanism by which rotenone induces neurodegeneration is not known. Evidence generated from in vitro and in vivo experiments points to the role of oxidative stress in PD.[17] Although L-Dopa is the most efficacious drug for reducing symptoms of PD, its continuous usage can result in dyskinesia and on-off phenomenon.[16]

Our study findings emphasized the effectiveness of SN in reducing oxidative stress and protecting against the degeneration of dopaminergic neurons. We noted significant enhancements in the climbing capabilities of *Drosophila* after the administration of SN. The increased climbing ability suggests that SN might have a positive impact on motor function, which is often severely compromised in PD. This development is encouraging, as movement impairments are a characteristic feature of Parkinson's disease and have a substantial effect on the patient's quality of life.

Sinomenine has demonstrated efficacy in PD and Alzheimer's disease models by suppressing microglial activation in vitro. SN might reduce inflammation-induced neurodegeneration in primary midbrain neuroglia culture utilizing LPS- and MPP+ mediated rat and mouse PD models. The focus for SN-mediated neuroprotection is the suppression of microglial PHOX activity, which is a subunit of NADPH oxidase. Inhibition of microglial PHOX activity is achieved by preventing the translocation of the cytosolic component p47phox to the plasma membrane, which is essential for extracellular ROS generation. Inhibiting PHOX results in decreased generation of additional pro-inflammatory mediators like intracellular ROS, TNF-alpha, NO, and PGE2.[18,19] The neuroprotective effects of SN studied against ischemic brain injury both in vivo and in vitro were investigated. SN exerted potent protective effects against ischemic brain injury when

administered before ischemia or even after the injury.[20]

In our study, it was observed that there was a decrease in oxidative stress in terms of MDA level in the Levodopa group followed by the SN high-dose group (0.25%). A study conducted by Abdul *et al.* on the SNCA-induced PD model of *Drosophila* also used MDA as an oxidative stress marker. They further proved that their study drug successfully reduced oxidative stress by reducing MDA levels.[21] Another study by Shirley Medina-Leendertz *et al.* also used MDA to check oxidative stress in the manganese-exposed*Drosophila* model of PD.[22]

To assess the neuroprotective impact of the experimental medications on a *drosophila* model of PD caused by rotenone, dopamine levels were also measured. It was observed that the Levodopa group and both the doses of SN (0.125% and 0.25%) showed a significant rise in dopamine levels as compared to disease control. Previous studies on genetic and rotenone-induced PD models of *Drosophila* also used dopamine assay to measure anti-parkinsonian effects.[23,24]

PD is characterized by a loss of dopaminergic neurons, which is responsible for the motor symptoms observed in patients. The increase in dopamine levels following SN treatment suggests that it might have a neuroprotective effect on dopaminergic neurons. This may be due to its impact on the preservation of dopamine-producing neurons or the improvement of dopamine production. Regardless, this observation shows significant potential for treatment interventions in PD.

Thus, the study unveils compelling insights into the impact of SN treatment on rotenone-induced PD. Not only did SN enhance climbing ability in *Drosophila*, but it also elevated dopamine levels in their brains. It also decreased the levels of malondialdehyde (MDA), a biomarker for oxidative stress that contributes to the development of Parkinson's disease by causing the destruction of dopaminergic neurons. This indicates that SN may be neuroprotective, probably by mitigating oxidative stress in the *Drosophila* model.

Employing the *Drosophila* model presents various advantages, including cost-effectiveness, accessibility, and a relatively short lifespan facilitating swift data acquisition. However, it's crucial to recognize that extrapolating these findings to human PD patients necessitates further exploration. Biological and genetic disparities between humans and *Drosophila* warrant cautious interpretation of the results concerning human health.

CONCLUSION

The findings of this study highlight the potential of Sinomenine as a therapeutic agent for Parkinson's disease. The observed improvements in climbing ability, reduction in brain MDA levels, and increase in dopamine levels suggest a multi-faceted protective effect against PD-like symptoms in a *Drosophila* model. Further research is needed to validate these promising findings and to determine the efficacy of Sinomenine as a treatment in Parkinson’s Disease.

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Conflict of Interests:

The authors declare that there is no conflict of interest.

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